THE DESIGN FEATURES AND PERFORMANCE OF A STATE-OF-THE-ART FULLY-AUTOMATED ANTI-MÜLLERIAN HORMONE IMMUNOASSAY FOR THE BECKMAN ACCESS FAMILY OF IMMUNOASSAY SYSTEMS

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Immunooassays for anti-Müllerian hormone (AMH) are considered a significant clinical tool to estimate an individual woman’s ovarian reserve. The following review discusses the design features and performance of the fully-automated Beckman Coulter Access AMH immunoassay.

Introduction

Beckman Coulter AMH assays have dominated research and clinical applications in the field of AMH testing since Beckman Coulter launched the Immunotech assay in 1999, resulting in several thousand related scientific papers. Many clinical applications\(^1\) have been identified, almost entirely using Beckman Coulter AMH assays. Figure 1 summarizes some of the key milestones in the Beckman AMH assay development program.

![Figure 1: Key Beckman Coulter milestones in development of AMH immunoassays](image)

Circulating molecular forms of AMH measured by Beckman Coulter assays

The main structural forms of human AMH are shown in Figure 2. The precursor non-bioactive form of AMH is known as proAMH. The dimeric N-terminal portion of proAMH is known as AMH\(_N\) (also known as the pro-region). The smaller dimeric C-terminal region (mature region) is known as AMH\(_C\)\(^2,3\). Secreted AMH is a homodimer with two identical polypeptide chains, each of 536 amino acids, each with a molecular weight of approximately 70 kDa with intra- and inter-chain disulfide bonds and glycosylation in the pro-region\(^4\). The bioactive form AMH\(_{N,C}\) is generated from proAMH by intracellular or extracellular cleavage with furin- or plasmin-type proteases.\(^2,3,4\) An additional potential furin cleavage site is found in human AMH at amino acid position 229 and forms of AMH that undergo cleavage at this site can be found in various recombinant AMH preparations\(^5,6\) but only occasionally in serum.\(^7\)

Pankhurst et al.\(^2\) demonstrated that both proAMH and AMH\(_{N,C}\) circulate in the blood in approximately equal amounts. No evidence has been found for the presence of AMH\(_N\) or AMH\(_C\) in the circulation as separate entities.\(^2\) The same group also demonstrated that the Beckman Coulter Gen II AMH ELISA measures both of these in a similar, if not identical, manner, but does not measure AMH\(_N\) or AMH\(_C\).\(^3\)
Figure 2. Main structural forms of AMH in the human circulation.

Current AMH immunoassays provide values that give high levels of linear correlation to each other, even though absolute values may vary. These comparisons have recently included the following: Beckman Coulter Gen II (2013 modification to include a premix protocol) with the Beckman Coulter Immunotech assay \( r = 0.98 \); Beckman Coulter Gen II (premix protocol) with the Beckman Coulter Access AMH assay \( r = 0.99 \). Earlier comparisons were conducted between the Immunotech assay and the Gen II assay \( r = 0.98 \) and of the Gen II assay with the prior Diagnostic Systems Laboratories (DSL) assays \( r = 0.97 \). These close correlations between results that were obtained with two different antibody pairs and three different assay formats suggest that, as far as clinical application is concerned, all assays essentially measure the same material and no assay is unusually susceptible to sample instability or variations in the glycosylation state of AMH. The report from Pankhurst el al. \(^2,3\) suggests that total AMH is being measured by all assays using the Gen II antibody pair. As AMH\(_{N,C}\) is the bioactive form of AMH\(^2,3,12\) it might be thought that the specific measurement of AMH\(_{N,C}\) forms would be essential or preferable to measuring total AMH levels. However, the finding in recent studies that the proportion of bioactive AMH is highly consistent indicates that it could be less significant that both ProAMH and AMH\(_{N,C}\) react equally to measure total AMH, as discussed by Pankhurst.\(^3\) For example, if one form gave a slightly lower signal mole for mole than the other, then this would simply reduce the overall signal slightly. If one patient had twice as much bioactive AMH as another, their apparent total signal would still double as the proportionate shares of signal from each form would both
double. It is only under circumstances where the ratio between the two forms varied widely that clinical decision-making and cut-offs might be affected. Despite these considerations, as stated it is likely that all the main immunoassays detect ProAMH and AMH\textsubscript{N,C} equally.

It is not known at this time whether the percentage of bioactive AMH varies in any disease states, or how it is affected by the myriad of variables known to affect total AMH levels, such as obesity, vitamin D levels and contraceptives, etc. In the evaluation of ovarian function, it is the constancy of total AMH through the cycle which has made AMH a valuable tool and every clinical application to date has been discovered using assays for total AMH. It appears likely that the clinical utility of AMH will continue to focus on the measurement of total AMH, but assays for bioactive AMH may be useful research tools. Future research will help to define the use of measurement of different forms of AMH.

**The choice of matrix for the Access AMH assay calibrators**

The matrix of an immunoassay is the milieu which contains the AMH calibration material. To perform its purpose, the matrix has to simulate the immunochemical and physical behaviour of authentic analyte-free human specimens. The matrix should be free of endogenous AMH in order to give consistently low batch-to-batch background levels. Mismatching between a matrix that may have residual AMH and analyte-free serum samples can result in falsely low results, and thus should be avoided.

Matrix with endogenous AMH can be heat-treated to remove unwanted endogenous AMH signal, a process which must be done thoroughly and reproducibly to ensure there is no variable batch-to-batch background that could impair assay sensitivity. However, some reactive AMH epitopes can survive heating and still react with the assay antibodies affecting the dose-response and introducing an undesirable complexity to a key assay component.

To reduce any such possible batch-to-batch problems, *the Access AMH assay uses a non-serum based calibrator matrix to avoid the endogenous AMH activity and to simulate analyte-free human serum*. The choice of this matrix, along with a small sample volume of 20 µl, is expected to ensure batch-to-batch consistency, in terms of assay performance and detection limitations with the Access assay. Additionally, the use of the synthetic matrix helps to provide a 90-day reconstituted stability at 2-10°C.\textsuperscript{9} The small assay volume of 20 µl also removes the need to have a serum-based matrix in order to replicate the physical characteristics of serum viscosity, ionic strength, etc.

**The antigen choice for the Access AMH assay calibrators and controls**

Calibration of AMH assays has been a major source of confusion for the clinical field.\textsuperscript{13,14} There are some potential drawbacks to using non-human antigen in an AMH assay for testing human samples.

There is no published evidence that bovine AMH, used in some commercial assays, is 100% cross-reactive with human AMH. To date, no recombinant or purified bovine AMH has been available to test the level of cross-reactivity in a direct fashion. It is thus possible that the association or dissociation rates of the capture or detection antibodies might differ when reacting with AMH from different species resulting in a dose-response relationship which differs between human AMH and the bovine calibrator. The low end of the assay may reflect this possible occurrence to the greatest extent.
The Access AMH assay was designed to eliminate the potential negative impacts of bovine serum by use of a synthetic calibrator matrix and by using recombinant human AMH in the calibrator.

In response to the current high level of clinical interest in AMH, several different potential recombinant calibration materials have been provided to NIBSC (National Institute of Biological Standards and Control) as candidates for an international AMH immunoassay reference material. NIBSC has an estimated completion and release date of 2016-2017. The track record and homogeneity of the Beckman Coulter recombinant AMH preparation (also used in the Immunotech AMH assay) and its stability to lyophilization should make it an ideal choice as an international reference standard for immunoassays of total AMH.

The AMH antigen in quality control materials should also behave in a similar manner to the AMH in native serum samples and allow for the assessment of true intra- and inter-assay variation. The Beckman Coulter AMH control kit has three controls that are human plasma spiked with recombinant human AMH (cat. no. B13129).

The working range of AMH assays

The Beckman Access AMH assay\textsuperscript{16} has a measuring range of approximately (0.02 to 24 ng/mL), which is the widest analytical measuring range of any current AMH immunoassays. This wide measuring range is largely due to the use of chemiluminescent detection in the automated assay.

There is now clear evidence that additional sensitivity is desirable for the assessment of AMH levels in individuals who are nearing menopause\textsuperscript{17} and to investigate residual ovarian reserve after chemotherapy.\textsuperscript{18} In one evaluation study using the Access AMH assay on the Access 2 system, LoD was tested using a protocol based on CLSI EP17-A2. This study determined the LoD for Access AMH to be 0.0049 ng/mL (0.035 pmol/L). A second study using the DxI system determined the LoD for Access AMH to be 0.0098 ng/mL (0.070 pmol/L).

LoQ for the Access AMH was tested using a protocol based on CLSI EP17-A2. Using the Access 2 system the study determined the LoQ for Access AMH to be 0.010 ng/mL (0.071 pmol/L) and using the DxI system in a separate study the LoQ was determined to be 0.011 ng/mL (0.079 pmol/L).

Whilst a working range up to approximately 24 ng/ml AMH is adequate for most IVF applications, some male paediatric samples (and those from patients with recurrent granulosa cell cancer) may need dilution, which is available with onboard dilution with the Beckman Coulter DxI system. The challenge in automating AMH assays for the most common applications has been to improve the assay sensitivity and increase the working range which has been accomplished with the automated Access AMH.

The specificity of the Access AMH assay

Immunoassays are very specific, but can be subject to cross reactants and interferences. Serum total protein concentrations are approximately 100 mg/ml, of which the main component is albumin. To achieve a specific detection limit of less than 0.02 ng/mL, the Access assay is required to measure AMH accurately in the presence of high excess of other proteins.
During the validation of the Access AMH assay, three members of the TGF-β family (inhibin A [100 ng/ml], activin A [16 µg/ml] and TGF-β1 [65 ng/ml]) were tested by Beckman Coulter and found to have no significant cross-reactivity. Two additional pieces of evidence support the specificity of the Access assay. In testing over 344 random (no exclusions for disease) postmenopausal serum greater than 93% samples were ≤ 0.02 ng/mL and greater than 97% were ≤ 0.08 ng/mL.

Despite their extensive use for clinical and basic research, there have been no reports of false evaluations with the Beckman Coulter DSL or Gen II AMH ELISAs and only two published reports of any false-positives with the Immunotech assay, which were confirmed as interfering antibodies using Scantibodies HBT tubes. The many correlations between different AMH immunoassays mentioned earlier suggest that any false-positive signals were likely to be small and infrequent in all assays, as outliers were rarely reported.

The data regarding serum AMH levels in the 344 postmenopausal women is indicative of the size and frequency of false-positive signals that are be expected in clinical practice, with these caveats: there is no information regarding the medical history of this panel, occupational exposure to animals, their exposure to antibodies for therapeutic or diagnostic purposes or any of the other factors that influence the development of heterophilic or rheumatoid antibodies. Many of these patients (median age 79) were two or three times the age of most of the women being tested clinically for AMH with a long lifetime of opportunity to acquire interfering antibodies. Additionally, some of the women may have had in the past, or be developing, granulosa cell cancer, and therefore the observed signal might be due to genuinely present AMH.

**The robustness of the Access AMH assay to false-negative interference**

A falsely lowered immunoassay interference may be exhibited if the sample contains a substance which interferes with or prevents the antigen-antibody sandwich formation. One example of interference is when complement activation occurs in the serum sample, which results in the loss of immunoassay signal from the analyte due to the deposition of complement component C3b on the capture antibody. Such interference has been reported previously and resolved in assays for other molecules. Such a problem was reported regarding the AMH Gen II assay in 2012 by Rustamov et al., who provided more extensive discussion in a subsequent paper. The problem manifested by increasing AMH upon storage for one week or more, serially diluted samples did not give quantitative results, and there was a downward drift in the median AMH values for different patient populations compared to the results from earlier versions of the AMH Gen II kit.

Initially, it was believed the apparent increases after certain storage conditions and the dilution anomalies were caused by the AMH molecule itself undergoing proteolysis or conformational changes. In fact, the dilution and storage issues were caused by the presence of active complement which decayed on storage. After investigating and validating a modified procedure, Beckman Coulter released a new version of the AMH Gen II assay in mid-2013, which involved premixing the sample and diluent before addition to the microplate. The modified method resolved the potential issue with complement interference and restores the satisfactory assay performance. Several successful reports have been published by customers using the new method.

An investigation into the nature of the complement interference was presented at European Society of Human Reproduction and Embryology (ESHRE) 2014. Both the AMH Gen II assay
and Access AMH assays can be used with fresh serum and plasma samples with no interference from complement, with good serial dilutions and recovery.

The stability of AMH in patient samples

AMH as measured with the Access AMH assay is now known to be extremely stable in serum under the normal range of conditions typically used for clinical sample collection and processing. In one study, it was found that samples are stable at room temperature for up to 24 hours either in separated serum or plasma or whole blood. AMH is stable in serum and plasma at 2 to 8°C for up to 6 days. For long-term storage, it is recommended to store at -20°C or -80°C. Additionally, data has shown that when using the Access AMH assay there is a negligible fall in measured AMH after whole blood samples were stored 5 days at room temperature, then separated, stored at 4°C and tested two days later.  

The automation of AMH assays

The limitations of manual ELISAs are well known, requiring users to follow kit instructions and display proficient manual pipetting skills. The current generation of clinical laboratorians is concerned with reducing labor costs and demands excellent levels of performance, ease of use, high throughput capabilities and affordability. They also need to report consistently reliable results to their physicians and patients. After 14 years of providing clinical results with manual AMH ELISAs, the field is now ready to move to state of the art testing options. Manual assays have enabled critical research to be performed and the clinical value of assays to be established, and the technical lessons learned during this process has benefitted the design of the novel state-of-the-art automated assays.

SUMMARY OF THE KEY FEATURES OF THE BECKMAN COULTER ACCESS AMH ASSAY

1. Measures “total AMH”: ProAMH (covalently intact), and bioactive AMH_{N,C} (after cleavage at amino acid 427).
2. Provides results that are in close agreement with the Immunotech and Modified Gen II assays.
3. Six concentrations of human recombinant AMH calibrators in a synthetic matrix with a 90-day shelf life after reconstitution.
4. Three levels lyophilised human recombinant AMH controls in processed human plasma.
5. Demonstrated limit of detection (LoD) of 0.0049 ng/mL and limit of quantitation (LoQ) of 0.010 ng/mL.
6. Approximate analytical routine measuring range: 0.02-24 ng/mL.
7. No observed interference by complement or cross-reactants.
8. Good dilution recovery and serial dilution capabilities.
9. High levels of stability of immunoreactive AMH in human sera and plasma.
10. Fully automated versions are available on all Access systems.
Figure 3. Design of the Beckman Access AMH Assay. Light emitted is directly proportional to AMH concentration

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